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Process Biochemistry 42 (2007) 1614–1619

Process Biochemistry

www.elsevier.com/locate/procbio

Simultaneous saccharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol☆

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Received 2 January 2007; received in revised form 9 August 2007; accepted 12 September 2007

Abstract

The effects of D-limonene concentration, enzyme loading, and pH on ethanol production from simultaneous saccharification and fermentation (SSF) of citrus peel waste by *Saccharomyces cerevisiae* were studied at 37 °C. Prior to SSF, citrus peel waste underwent a steam explosion process to remove more than 90% of the initial D-limonene present in the peel waste. D-Limonene is known to inhibit yeast growth and experiments were performed where D-limonene was added back to peel to determine threshold inhibition amounts. Ethanol concentrations after 24 h were reduced in fermentations with initial D-limonene concentrations greater than or equal to 0.33% (v/v) and final (24 h) D-limonene concentrations greater than or equal to 0.14% (v/v). Ethanol production was reduced when enzyme loadings were (IU or FPU/g peel dry solids) less than 25, pectinase; 0.02, cellulase; and 13, beta-glucosidase. Ethanol production was greatest when the initial pH of the peel waste was adjusted to 6.0.

Keywords: Ethanol; Citrus; Yeast; Fermentation; Enzymes; D-Limonene

1. Introduction

Demand for fuels produced from renewable resources has increased in recent years due to increased prices for oil, concerns about greenhouse gas production, and increasing reliability on foreign sources of energy in the US [1]. One of the most prevalent renewable energy sources in the US is ethanol. Ethanol is generally produced from corn (maize) in the US with a total production at the end of 2005 of 1.5×10^{10} l/year with an additional 5.7×10^{9} l of capacity under construction [2]. Many states in the US have little or no fuel ethanol production, making a transition to ethanol-blended gasoline dependent on importing ethanol from other states or countries. Development of local feedstocks to produce ethanol in these states would increase ethanol supply and encourage use of ethanol-blended gasoline in parts of the country where it is not currently available. The state of Florida, for example, had no fuel ethanol plants as of December 2006. A local feedstock produced in Florida for ethanol production would allow the development of a local ethanol industry that would increase the use of renewable fuels and decrease MTBE use and it potential hazards to groundwater.

A potential Florida feedstock for ethanol production is citrus peel waste (CPW). CPW consists of the peel, segment membranes, and seeds left over after oranges, grapefruit, and other citrus fruits have been juiced. Over the last 10 years, an average of over 4.5×10^{9} kg of CPW were produced annually from the processing of oranges and grapefruits in Florida for production of juice products. [3]. Citrus juice processors generally dry and pelletize this waste into cattle feed called citrus pulp pellets (CPP), which is sold at a loss to the processor. Some small processors cannot afford to invest capital in the equipment needed to produce CPP and must pay haulers to take CPW away from their facility for disposal.

In previous studies, CPW was successfully hydrolyzed by enzymes to sugars and subsequently fermented to ethanol by

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1359-5113/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2007.09.006

Saccharomyces cerevisiae yeast and *Escherichia coil* K011 bacteria [4–6]. This study applies simultaneous saccharification and fermentation (SSF) techniques to the production of ethanol from CPW. SSF combines enzymatic hydrolysis with fermentation in the same vessel at the same time. Enzymes hydrolyze polysaccharides into sugars that are immediately consumed by yeast to produce ethanol. SSF increases hydrolysis rates by reducing product inhibition of enzymes and reduces tank usage by combining the saccharification and fermentation tanks into one tank. SSF is widely used in the dry grind corn ethanol industry [7]; however, CPW has to be pretreated to remove D-limonene below inhibitory levels for yeasts [6,8–11] prior to SSF.

Steam explosion is a potential pretreatment for CPW to increase enzyme accessibility to cell wall polysaccharides while removing D-limonene. Steam explosion has been employed on a variety of cellulosic feedstocks to prepare them for subsequent enzymatic hydrolysis and sugar fermentation. Steam explosion is a process by which pressurized steam is applied to cellulosic biomass in a pressure reactor. After a set reaction time, some of the steam is vented to quickly reduce the pressure in the reactor, thus causing the water in the biomass to rapidly decompress, thus causing disruption of cell walls. Additionally, hemicellulose in the biomass is hydrolyzed, probably by organic acids [12]. After steam pretreatment, the surface area of the biomass is increased and cellulose is more accessible to enzymatic attack.

In this study, we applied a modified steam explosion process to volatilize and remove peel oil for CPW and prepare CPW for SSF. The objectives of this study were to evaluate the effect of D-limonene concentration, enzyme loading, and initial pH on the amount of ethanol produced by *S. cerevisiae* during SSF.

2. Materials and methods

2.1. Peel pretreatment and analyses

Citrus peel waste (peel, seeds, and membranes) was collected from commercial orange juice facilities. Peel used for the effect of D-limonene and effect of enzyme loading experiments contained 0.8% D-limonene, a monoterpene that inhibits yeast and other microorganisms. Orange peel oil contains more than 90% (w/w) D-limonene [13]. CPW used for effect of initial pH experiments contained 1.6% (v/w) D-limonene. CPW that contained 0.8% (v/w) D-limonene was collected from a facility that recovered peel oil during juice extraction; all other CPW used was collected from a facility without oil recovery. D-Limonene content of the CPW before and after pretreatment was determined by the Scott oil method [14].

The CPW was pretreated with live steam in a continuous tube reactor at 150–160 °C for approximately 2–4 min [15]. The hot material was flashed to a cyclone where most of D-limonene was stripped by escaping steam. The pretreated waste was collected from the bottom of the cyclone and frozen. Frozen, pretreated CPW was stored at −20 °C and thawed as needed for analyses or SSF experiments. After D-limonene removal, D-limonene content of CPW used for the effect of D-limonene and effect of enzyme loading experiments was 0.08% (v/w) and dry matter content was 18.58% (w/w). D-Limonene content of CPW used for the effect of pH study was 0.16% (v/w) and dry matter content was 23.37% (w/w). CPW dry matter content was determined by drying at 70 °C for 20 h followed by vacuum drying at 70 °C for 1 h. CPW soluble carbohydrate content prior to SSF was determined by water extraction as previously described [16].

2.2. SSF procedure

SSF experiments were carried out in 250 ml amber glass bottles with a stopper and a one-way valve to allow the release of carbon dioxide produced during fermentation. Treated CPW (100 g) was added to each bottle. Pectinase (Pectinex Ultra SP), cellulase (Celluclast 1.5 L) and beta-glucosidase (Novozym 188) preparations were added to each bottle. All enzymes were obtained from Novozymes A/S (Bagsvaerd, Denmark). *S. cerevisiae* in the form of active dry yeast (Fleischmann, St. Louis, MO, USA) was also added to each bottle as described previously to obtain an initial yeast concentration of 0.7 g cells/100 g CPW [5]. Initial pH and the amounts of enzymes varied, as will be described later. Bottles were rotated at 10–12 rpm at 37 °C. Bottles were then frozen until analyses were completed. Ethanol, glucose, and galacturonic acid (GA) concentrations were determined by HPLC on an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C at 0.5 ml/min with 0.01N sulfuric acid as the eluent. The pH of CPW was measured using a handheld ISFET pH meter (IQ Scientific Instruments, Carlsbad, CA, USA).

2.3. Effect of D-limonene

Pectinase, cellulase and beta-glucosidase were added to the CPW at the loadings of 1.7, 1.4 and 1.5 mg protein/g peel solids, respectively [5]. Calcium carbonate was added to the peel to increase pH to 4.8. Bottles were rotated at 10–12 rpm for 3 h at 37 °C to liquefy the sample. After 3 h of hydrolysis, 10 ml of yeast starter solution containing 0.7 g *S. cerevisiae* yeast, 0.1 g glucose, 0.1 g peptone, and 0.1 g yeast extract was added to each bottle. Orange peel oil containing 95% (v/w) D-limonene was also added to each bottle to achieve D-limonene contents in CPW of 0.08, 0.13, 0.18, 0.23, 0.28, 0.33, and 0.43% (v/w). The bottles continued rotating at 10–12 rpm for an additional 24 h at 37 °C. The bottles were then frozen until analyses were completed. Ethanol concentrations for each SSF experiment were determined by HPLC as described earlier. D-Limonene was determined both prior to and after SSF.

2.4. Effect of enzyme loading

Pectinase loadings ranging from 0 to 396 IU/g CPW solids, cellulase loadings ranging from 0 to 0.18 FPU/g peel solids were tested, and beta-galactosidase loadings ranging from 0 to 52.3 IU/g peel solids. The initial pH was adjusted to 5.0 with $CaCO_3$. Activities for pectinase and cellulase were 8740 IU/ml (233 IU/mg protein) and 10.3 FPU/ml (0.126 FPU/mg protein), respectively [17]. Beta-galactosidase activity was reported by the manufacturer to be 237.5 IU/ml (1.32 IU/mg protein).

2.5. Effect of initial pH

Initial pH of the pretreated CPW from the commercial juice facility was between 4.3 and 4.4. Calcium carbonate was added to the peel before the fermentation to increase pH to 5.0, 5.6, or 6.0. No additional calcium carbonate was added to the peel during fermentation. Ethanol, glucose, and galacturonic concentrations and pH were determined for each fermentation after 24 and 48 h by HPLC as described earlier.

2.6. Statistics

For all experiments, analysis of variance (ANOVA) was calculated ($p < 0.05$) using the mixed procedure in SAS (Release 9.1, Cary, NC) and differences among means were calculated using Fisher's protected least significant difference test ($p < 0.05$). For the initial D-limonene experiment, initial D-limonene content was the dependent variable and final ethanol and D-limonene concentrations were the independent variables. For initial pH tests, initial pH was the dependent variable and 24 and 48 h ethanol concentrations were the independent variables. For enzyme loading tests, pectinase, cellulase, and beta-glucosidase loadings were the dependent variables and final ethanol concentration was the independent variable. Main effects, two-way, and three-way interactions were tested for enzyme loading tests. The carbohydrate content CPW for each experiment was assumed to be the same for each SSF run.

3. Results and discussion

3.1. Effect of D-limonene

The soluble carbohydrate content after pretreatment for CPW used in the D-limonene and enzyme loading experiments was (%dry weight (dw)) 10.74, fructose; 10.16, glucose; 0.10, galactose; and 10.91, sucrose, (CPW dry matter was 18.58%, w/w). Assuming ethanol production of 90% theoretical by *S. cerevisiae*, an ethanol concentration of 27.21 g/l would be expected without SSF. The maximum ethanol concentration achieved in the effect of D-limonene experiment was 39.03 g/l (Fig. 1, 0.08%, v/w, D-limonene), which corresponds to an increase of 25.75 g/l in carbohydrates fermentable by *S. cerevisiae*, or 13.86% dw of the CPW. This increase is similar to what was observed using CPW from oranges in a previous study [16].

Initial D-limonene content had an effect on both ethanol production and final D-limonene content ($p < 0.05$). Ethanol concentrations in the fermented hydrolyzates after 24 h with initial D-limonene contents of 0.08, 0.13, 0.18, 0.23, and 0.28% (v/w) were not different from one another ($p < 0.05$) (Fig. 1). Ethanol concentrations of fermented hydrolyzates with initial D-limonene contents of 0.33 and 0.43% (v/w) were less than other experiments ($p < 0.05$). Fermentation experiments with initial D-limonene contents of 0.08% (v/w) and 0.13% (v/w) had the lowest final D-limonene contents, and experiments with an initial D-limonene content of 0.43% (v/w) had the highest final D-limonene content ($p < 0.05$) (Fig. 1). Fig. 2 shows the relationship between final D-limonene content of fermented hydrolyzates and ethanol concentration. Inhibition of ethanol production was observed at concentrations greater than or equal to 0.12% (v/w), which is similar to values reported previously [6,10].

Previous studies have observed inhibition of microbial growth at concentrations between 0.05 and 0.15% (v/w) [5,8,10,11]. In our study, a fairly large inoculum (7 g cells/L) was used, which

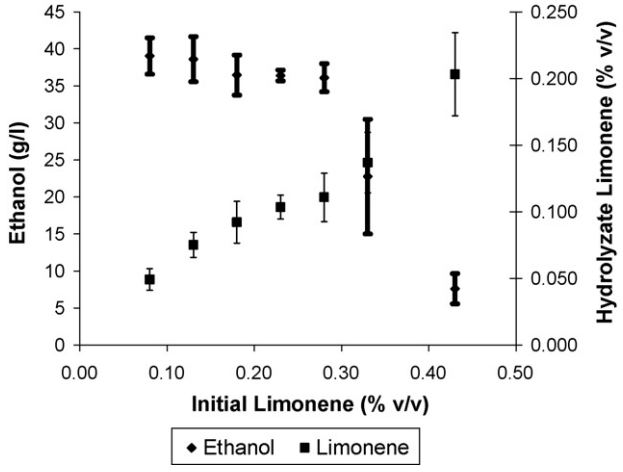

Fig. 1. Hydrolyzate ethanol and limonene concentrations after 24 h SSF using citrus peel waste with varying initial limonene concentrations (error bars are ±1 standard deviation, mean of 3 SSFs except 0.28 and 0.33% (v/w), which are the mean of 4 SSFs).

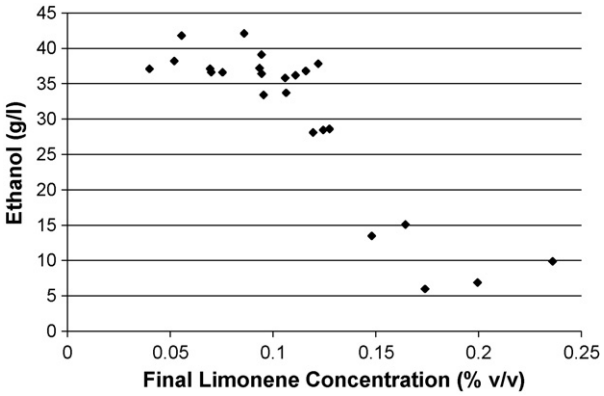

Fig. 2. Ethanol yields related to the limonene concentration after each SSF.

was greater than the studies previously mentioned with the exception of Grohmann et al. [5]. Larger inoculum size probably enabled the yeast to overcome inhibitory effects due to D-limonene, which resulted in a greater minimum inhibitory concentration. D-Limonene's inhibitory effects on *S. cerevisiae* have been attributed to its disruption of the cellular membrane disruption causing cellular components to leak out of the cell as well as disruption H^+ and K^+ transport energized by glycolysis [18–20]. It is not known how ethanol production progressed over the course of the fermentation since sampling was not done except at the end of 24 h. Since sugars contained in hydrolyzed CPW can be completely consumed in 7–10 h [5], it is likely that fermentations with greater initial D-limonene contents were inhibited initially, but as D-limonene was vented from the fermentation bottles or converted by yeast to less toxic compounds, inhibition decreased. In a study with *S. cerevisiae* and *Kluyveromyces marxianus*, lag times of >24 h were observed in fermentations containing 0.05–0.20% (v/w) orange peel oil before sugars were completely fermented to ethanol [11].

Between 38.5 and 60.4% of the limonene present at the beginning of SSF was removed and/or converted to other products during SSF (Fig. 1). Several yeast species are known to be able to convert D-limonene to such products as isopiperitone, *trans*-1,2-dihydroxylimonene, and perillic acid [21]; however, all yeast biotransformations of limonene thus far have been found to be catalyzed by monooxygenases, which require oxygen [22]. Since SSF was carried out under anaerobic conditions, an oxidation of limonene catalyzed by a monooxidase is unlikely. To our knowledge, *S. cerevisiae* has not been observed to convert D-limonene into other products. Further investigation is needed to determine the fate of D-limonene during anaerobic fermentation by *S. cerevisiae* and other yeast.

3.2. Effect of enzyme loading

During the enzyme loading experiment, an increase in carbohydrates fermentable by *S. cerevisiae* similar to the D-limonene experiment was observed, with the maximum ethanol concentration achieved being 39.60 g/l (Table 1, 297 IU/g dm). This was expected since the CPW used for both experiments was from the same batch of material.

Table 1
Ethanol concentrations in simultaneous saccharification and fermentations containing various amounts of hydrolysis enzymes (values with the same letter are not significantly different, $p > 0.05$)

Pectinase activity (IU/g CPW dry matter)	Ethanol (g/l)	BG activity (IU/g CPW dry matter)	Ethanol (g/l)	Cellulase activity (FPU/g CPW dry matter)	Ethanol (g/l)
0	26.97[a]	0	30.11[a]	0.00	24.00[a]
9	30.15[b]	5	29.67[a]	0.01	32.82[b]
19	31.05[b]	13	37.71[b]	0.02	38.05[c]
25	37.20[c]	26	38.68[b]	0.04	36.34[c]
49	37.80[c]	52	37.95[b]	0.09	38.00[c]
74	38.85[c]			0.18	37.48[c]
99	36.98[c]				
198	37.40[c]				
297	39.60[c]				

Ethanol concentration after SSF was reduced when pectinase loadings were less than 25 IU/g peel solids, cellulase loadings were less than 0.02 FPU/g peel solids, and beta-galactosidase loadings were less than 13 IU/g peel solids ($p < 0.05$) (Table 1). Loadings of 26.5 IU/g peel solids for pectinase and 0.27 FPU/g peel solids for cellulase were used in a previous study that used pectinase and cellulase enzymes to hydrolyze raw Valencia orange peel before fermenting the hydrolyzate with *S. cerevisiae* [5]. D-Limonene was removed in that study by filtering the hydrolyzate to remove solids that were later found to contain most of the D-limonene present after hydrolysis. Utilization of SSF techniques combined with D-limonene stripping and steam explosion reduced pectinase loading by 6% and cellulase loading by 92% as compared to the previous study [5].

These qualitative observations relate well to the viscosity measurements that were conducted on CPW hydrolyzed by a similar enzyme preparation in a previous study [23]. The pretreatment greatly increased CPW surface area, allowing the hydrolyzing enzymes to work more effectively on pectin and cellulose. Also, SSF probably contributed to reduction of enzyme use over previous studies due to decreased product inhibition of cellulase. It is not known if reduction of D-limonene contributed to reduction in enzyme usage.

3.3. Effect of pH

The soluble carbohydrate content for CPW used in the pH experiment was (%dw) 6.96, fructose; 7.14, glucose; and 13.48, sucrose, which, assuming 90% conversion of carbohydrate to ethanol by *S. cerevisiae*, corresponds to a theoretical ethanol concentration of 29.58 g/l (CPW dry matter was 23.37%, w/w). The maximum ethanol concentration achieved in the effect of D-limonene experiment was 36.10 g/l with 0.45 g/l glucose unconsumed (Figs. 3 and 4), an increase in fermentable carbohydrates of 14.2 g/l or 6.08% dw CPW. It is not known why the increase in fermentable sugar was less than that observed with the other batch of CPW used in the previous experiments. This CPW had a greater dry matter content, which may reduced the effectiveness of the pretreatment and/or the hydrolysis due to less water being available.

Initial pH had an effect on the ethanol produced at both 24 and 48 h ($p < 0.05$) (Fig. 3). Fermentations at initial pH 6.0 produced the most ethanol after 24 and 48 h, and fermentations at initial pH 4.4 produced the least ethanol at 24 and 48 h ($p < 0.05$). Ethanol concentrations at initial pH 5.0 and 5.6 were not different from each other ($p > 0.05$), but were less than pH 6.0 and greater than pH 4.4. Ethanol concentrations at 24 and 48 h were similar, indicating fermentations were complete at or before 24 h. Glucose and GA concentrations continued to increase from 24 to 48 h, indicating continued hydrolysis. Glucose and GA concentrations increased as pH decreased, indicating that pectinase enzyme was more effective at reduced pH (Fig. 4). This was expected since the optimum pH for the commercial pectinase preparation used in the study was 3.8, as reported by the manufacturer. GA concentration after fermentation is greater than glucose because *S. cerevisiae* cannot utilize GA. Residual glucose concentration increased as pH decreased, which followed the trend in ethanol concentration (Fig. 4). Increased glucose concentrations are due to the inability of yeast to ferment glucose at lower pH. Final pH after 48 h in fermentations with initial pH 4.4 was 3.3, which was the lowest pH of all the fermentations. Fermentations with initial pH of 5.0 and 5.6 had similar final pH values of 3.7 and 3.75, respectively. Fermentations with initial pH of 6.0 had the highest final pH of 4.9. Final pH correlated linearly with GA and glucose concentration in the fermentation slurry ($R^2 = 0.99$ and 0.97, respectively) (Fig. 4).

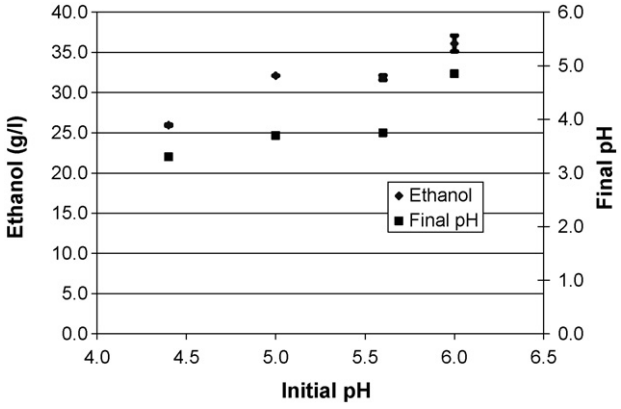

Fig. 3. Ethanol, glucose and GA concentrations and final pH values from SSF of orange peel waste at varying initial pH values (error bars are ±1 standard deviation, mean of 2 SSFs).

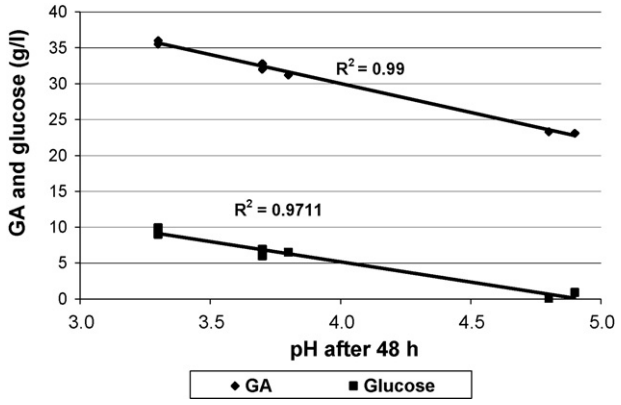

Fig. 4. GA and glucose concentrations at various pH after 48 h *S. cerevisiae* fermentations.

S. cerevisiae have been reported to increase ethanol production at pH 5.0 and 5.5 as opposed to pH 4.0 and 4.5 [24], and its optimum pH is from 5.0 to 5.2 [25]. Weak acids have been shown to inhibit *S. cerevisiae* growth at lower pH due to an increase in undissociated acids [26]. Several weak organic acids, such as acetic, malic, malonic, lactic, and citric acids, are known to be present in citrus peel [27], though in a recent report, citric acid, one of the more abundant acids in citrus peel, was found to increase ethanol yield with decreasing pH in the range of pH 3.0–4.5 [26]. The decrease in ethanol with decreasing pH observed here indicates that the inhibitory effect with decreasing pH of most organic acids in CPW was greater than the beneficial effect on ethanol production due to citric acid. The use of 37 °C as a fermentation temperature, which was a chosen as a compromise for increasing the activity of hydrolysis enzymes while allowing *S. cerevisiae* to still ferment sugars, also may have contributed to the decrease in ethanol at lower pH. The ideal temperature for most strains of *S. cerevisiae* is approximately 30 °C. Greater temperatures can stress the yeast and make them more susceptible to other stresses such as low pH and ethanol, especially at ethanol concentrations greater than 3% (w/v) [28].

4. Conclusion

CPW was treated with steam to remove D-limonene and sterilize and soften the peel waste prior to SSF by *S. cerevisiae*, which removed more than 90% of the initial D-limonene present in the peel waste. Ethanol concentrations after 24 h were reduced in fermentations with initial D-limonene concentrations greater than or equal to 0.33% (v/w) and final (24 h) D-limonene concentrations greater than or equal to 0.14% (v/w). Ethanol production were reduced at pectinase loadings less than 25 IU/g peel dry matter, cellulase loadings less than 0.02 FPU/g peel dry matter, and beta-glucosidase loadings less than 13 IU/g peel dry matter. Fermentations with an initial pH of 6.0 produced more ethanol than did fermentations with initial pH of 5.6, 5.0, and 4.4. The pH after 48 h of fermentations with an initial pH of 6.0 was 4.9, which is ideal for both *S. cerevisiae* and the cellulase enzyme used in the study.

Acknowledgements

The authors would like to acknowledge Renewable Spirits, LLC for their support of this project and Sandra Matlack for her assistance on analyzing samples.

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